

# Monovalent cation dependence and preference of GHKL ATPases and kinases<sup>1</sup>

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**Abstract** The GHKL phosphotransferase superfamily, characterized by four sequence motifs that form the ATP-binding site, consists of the ATPase domains of type II DNA topoisomerases, Hsp90, and MutL, and bacterial and mitochondrial protein kinases. In addition to a magnesium ion, which is essential for catalysis, a potassium ion bound adjacent to the triphosphate moiety of ATP in a rat mitochondrial protein kinase, BCK (branched-chain  $\alpha$ -ketoacid dehydrogenase kinase), has been shown to be indispensable for nucleotide binding and hydrolysis. Using X-ray crystallographic, biochemical, and genetic analyses, we find that the monovalent cation-binding site is conserved in MutL, but both Na<sup>+</sup> and K<sup>+</sup> support the MutL ATPase activity. When Ala100 of MutL is substituted by proline, mimicking the K<sup>+</sup>-binding environment in BCK, the mutant MutL protein becomes exclusively dependent on Na<sup>+</sup> for the ATPase activity. The coordination of this Na<sup>+</sup> ion is identical to that of the K<sup>+</sup> ion in BCK and involves four carbonyl oxygen atoms emanating from the hinges of the ATP lid and a non-bridging oxygen of the bound nucleotide. A similar monovalent cation-binding site is found in DNA gyrase with additional coordination by a serine side chain. The conserved and protein-specific monovalent cation-binding site is unique to the GHKL superfamily and probably essential for both ATPase and kinase activity. Dependence on different monovalent cations for catalysis may be exploited for future drug design specifically targeting each individual member of the GHKL superfamily.

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**Key words:** GHKL ATPase and kinase;  
 Monovalent cation; Active site;  
 Catalysis

## 1. Introduction

The GHKL phosphotransferase superfamily consists of type II DNA topoisomerases (represented by gyrase), Hsp90, bacterial histidine and mitochondrial serine protein

kinases, and the DNA mismatch repair protein MutL [1–4]. Members of this superfamily share four conserved sequence motifs essential for ATP binding that are unrelated to the Walker A and Walker B motifs present in many other ATPases [5]. Crystal structures of the ATPase fragment of gyrase (GyrB) [6], the ATP-binding fragment of Hsp90 [7], bacterial histidine kinase (CheA) [8], mitochondrial branched-chain  $\alpha$ -ketoacid dehydrogenase kinase (BCK) [9] and the ATPase fragment of bacterial MutL [10] and its human homolog (PMS2) [11] reveal that these four sequence motifs form a conserved ATP-binding pocket in a similarly folded structural domain. GHKL ATPases play crucial roles in DNA metabolism, protein folding and signal transduction. A number of commonly used antibiotic and anticancer drugs, including novobiocin, coumarin, geldanamycin and radicicol, target the ATP-binding sites of topoisomerase II and Hsp90 and inhibit the ATPase activity of these enzymes [12–15]. In vitro tests indicate that several drugs are cross-reactive due to the similarity of the ATP-binding pocket and may affect multiple members of this superfamily in vivo [16,17]. A challenge is to explore unique structural features of these enzymes and design drugs highly specific and devoid of cross-reactivity.

In all crystal structures of the GHKL proteins, three of four conserved sequence motifs (I, II and IV) form a concave ATP-binding pocket, while motif III, also known as F-G2 motifs [1], forms a flexible lid that encloses the bound ATP. Within the ATP-binding pocket, a divalent magnesium ion essential for the ATPase and kinase activities is coordinated by an absolutely conserved asparagine side chain from motif I (the N motif) and the triphosphate moiety of the nucleotide [6–11,18]. BCK, a mitochondrial serine protein kinase that regulates the oxidative decarboxylation of  $\alpha$ -ketoacids derived from branched-chain amino acids requires potassium in addition to magnesium for ATP binding and hydrolysis, however [9]. Superposition of the crystal structures of the N-terminal ATPase fragment of MutL (LN40) and BCK shows that the ATP-binding sites in these two enzymes are structurally very similar. In the MutL crystal structure, two water molecules were initially assigned to the site equivalent to that occupied by K<sup>+</sup> in BCK [14]. One of these water molecules is coordinated by four carbonyl oxygen atoms and may in fact be a monovalent cation. We therefore carried out structural and biochemical analysis of the monovalent cation dependence of the ATPase activity of both wild-type (WT) MutL and a mutant that contains an alanine to proline substitution, which renders the active site of the mutant MutL structurally more similar to that of BCK.

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<sup>1</sup> Coordinates: atomic coordinates and structure factors of the LN40–ADPnP(Rb), LN40–ADPnP(K) and LN40(A100P)–ADPnP complex have been deposited with the Protein Data Bank (accession codes 1NHJ, 1NHI and 1NHJ).

## 2. Materials and methods

### 2.1. Mutagenesis

The A100P mutant mutL (plasmid pWY1381) was derived from pTX418 [19] encoding WT mutL using a QuikChange Site-directed Mutagenesis kit (Stratagene). The sequence of the mutant was verified using a PRISM-310 (ABI) DNA sequencer.

### 2.2. Purification, crystallization and structural analyses

The N-terminal 40 kDa fragment of WT MutL (LN40) and the A100P mutant (LN40(A100P)) were purified as described previously [10,16]. To prepare the rubidium complex, LN40–ADPnP crystals were grown in a buffer containing  $\text{Li}_2\text{SO}_4$  [10], soaked for 30 min in a solution containing 100 mM Tris (pH 8.4), 1 M RbCl, 20% (w/v) PEG4000, 5 mM  $\text{MgCl}_2$ , 0.5 mM ADPnP, 1 mM EDTA and 1 mM dithiothreitol (DTT), cryo-protected by 15% (v/v) ethylene glycol and flash-cooled in liquid propane. Crystals containing  $\text{K}^+$  were grown from a solution composed of 100 mM Tris (pH 8.2), 150 mM  $(\text{NH}_4)_2\text{SO}_4$ , 50 mM KCl, 24% (w/v) PEG4000, 5 mM  $\text{MgCl}_2$ , 1 mM EDTA and 1 mM DTT at 20°C by micro-seeding and soaked in a solution containing 100 mM Tris pH 8.2, 0.4 M KCl, 0.05 M  $(\text{NH}_4)_2\text{SO}_4$ , 28% (w/v) PEG4000, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 1 mM EDTA, 0.5 mM ADPnP, 15% (v/v) ethylene glycol for 30 min. Crystals of the LN40(A100P) mutant protein were prepared as the WT LN40. The mutant protein was incubated with ADPnP and  $\text{Mg}^{2+}$ , exchanged buffer into KCl using a size exclusion column [10,16] and crystallized in the presence of KCl.

X-ray data were collected using an RU200 Rigaku X-ray generator. The WT LN40 crystals soaked in RbCl diffracted X-rays to 2.4 Å, KCl-soaked crystals diffracted to 2.0 Å, and the crystal of the LN40(A100P) mutant protein diffracted to 2.3 Å resolution. All crystals were of orthorhombic space group and isomorphous to the original LN40–ADPnP crystallized in  $\text{Li}_2\text{SO}_4$  [10]. Oscillation through a 60° range yielded diffraction data of 95% completeness. An additional 60° of data were collected in the inverse-beam geometry to measure the anomalous scattering due to Rb, K, S or P. Diffraction data were processed using HKL [20] (Table 1). Structural models were generated using O [21] and refined using procedures in CNS [22] (Table 1). Anomalous difference maps for each dataset were calculated using the CCP4 package [23].

### 2.3. ATPase activity assay

The full-length WT and mutant MutL (A100P) proteins were extensively dialyzed and concentrated to 5–10 mg/ml in buffers containing 20 mM Tris (pH 8.0), 1 mM DTT and 300 mM XCl (X = Li, Na, K, Rb, Cs) using Centricon-30 devices (Amicon). Subsequently, the effect of monovalent cations on the ATPase activity was determined at room temperature in 15 µl reactions containing 4 µg (6.7 µM) protein, 20 mM Tris (pH 8.0), 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 100–1000 mM XCl and 200 µM  $\alpha$ -labeled [ $^{32}\text{P}$ ]ATP. Hydrolysis products were separated on thin layer chromatography plates developed in 0.75 M  $\text{KH}_2\text{PO}_4$  and quantified using a TYPHOON 8600 Phosphor-Imaging Plate System (Molecular Dynamics).

## 3. Results and discussion

### 3.1. WT MutL is insensitive to the type and concentration of monovalent cation

The ATPase activity of WT MutL was tested in the presence of 50–1000 mM LiCl, NaCl, KCl, RbCl or CsCl after extensive dialysis of the protein into buffers containing a specific salt. We found that ATP hydrolysis occurred in the presence of all salts except for LiCl (Fig. 1a) and that the rate of ATP hydrolysis is about 1.5 to three times higher with NaCl than with any other salt. The catalytic rate is reduced by a factor of two when the salt concentrations are increased from 50 mM to 1000 mM (Fig. 1a), and the reduction is most dramatic between the salt concentrations of 50 and 150 mM. The reduced ATPase activity at higher salt concentrations may be explained by the reduced ATP binding due to increased ionic interactions in solution as predicted by the Debye–Huckel theory.

At the site where  $\text{K}^+$  is found in BCK [9], two water molecules were originally placed in the MutL structure determined from crystals grown in the presence of 200 mM  $\text{Li}_2\text{SO}_4$  [10]. To compare the structures of MutL and BCK and investigate binding of monovalent cations at atomic resolution, we soaked the native LN40–ADPnP complex crystals in a solution containing 1 M RbCl and found the relatively large and electron dense  $\text{Rb}^+$  ion replacing both water molecules near the ADPnP at a site similar to the  $\text{K}^+$  in BCK. When examining anomalous diffraction of LN40–ADPnP crystals soaked in KCl at the  $\text{CuK}\alpha$  edge at 2.0 Å resolution, we found that a potassium ion replaced one of the two water molecules (Fig. 2a). The  $\text{K}^+$  ion is coordinated by four carbonyl oxygen atoms (from residues 70, 71, 73 and 76) that emanate from a tight turn following an  $\alpha$ -helix at the N-terminal hinge of the ATP lid (Fig. 3). The carbonyl groups chelating the monovalent cation in BCK are located in the same secondary structure elements (residues 298, 300, 303 and 337) as in MutL, and three of the four carbonyl oxygen atoms are superimposable (Fig. 3). The  $\text{K}^+$  ion in BCK directly interacts with a non-bridging oxygen of the  $\alpha$ -phosphate of ATP $\gamma$ S in BCK (Fig. 2b). In contrast, the  $\text{K}^+$  ion in MutL does not directly contact ADPnP, and a water molecule provides the fifth ligand instead (Fig. 2a). The monovalent cation apparently stabilizes the ATP lid in both BCK and MutL. We suspect

Table 1  
Data collection and refinement

	LN40–ADPnP– $\text{Rb}^+$	LN40–ADPnP– $\text{K}^+$	LN40/A100P–ADPnP– $\text{Na}^+$
Data collection			
Space group		I222	
Cell constants (Å)	61.7, 71.2, 189.6	61.9, 71.4, 190.3	62.3, 72.6, 190.8
Diffraction data <sup>a</sup> (Å)	20.0–2.4 (2.44–2.40)	20.0–2.0 (2.03–2.00)	20.0–2.3 (2.34–2.30)
Completeness <sup>a</sup> (%)	93.3 (92.1)	95.6 (92.0)	93.6 (93.5)
$R_{\text{merge}}$ <sup>a</sup> (%)	6.1 (28.6)	4.3 (13.9)	5.4 (49.0)
Refinement			
Protein atoms	2588	2618	2620
Solvent and ligand atoms	272	229	271
Unique reflections	29493	51448	33550
$R$ and ( $R_{\text{free}}$ )	20.2% (24.9%)	20.8% (22.9%)	21.7% (26.6%)
Rmsd on bond length (Å)	0.005	0.005	0.006
Rmsd on bond angle	1.126°	1.117°	1.222°
Average $B$ of MC <sup>b</sup> (Å <sup>2</sup> )	34.0 (1.3)	33.4 (1.4)	45.9 (1.3)
Average $B$ of SC <sup>1b</sup> (Å <sup>2</sup> )	37.4 (2.1)	37.6 (2.2)	48.5 (2.1)

<sup>a</sup>Data of the highest resolution bin are shown in parentheses.

<sup>b</sup>MC, main chain atoms; SC, side chain atoms. Numbers in parentheses are the rmsd (root mean square deviation) values.

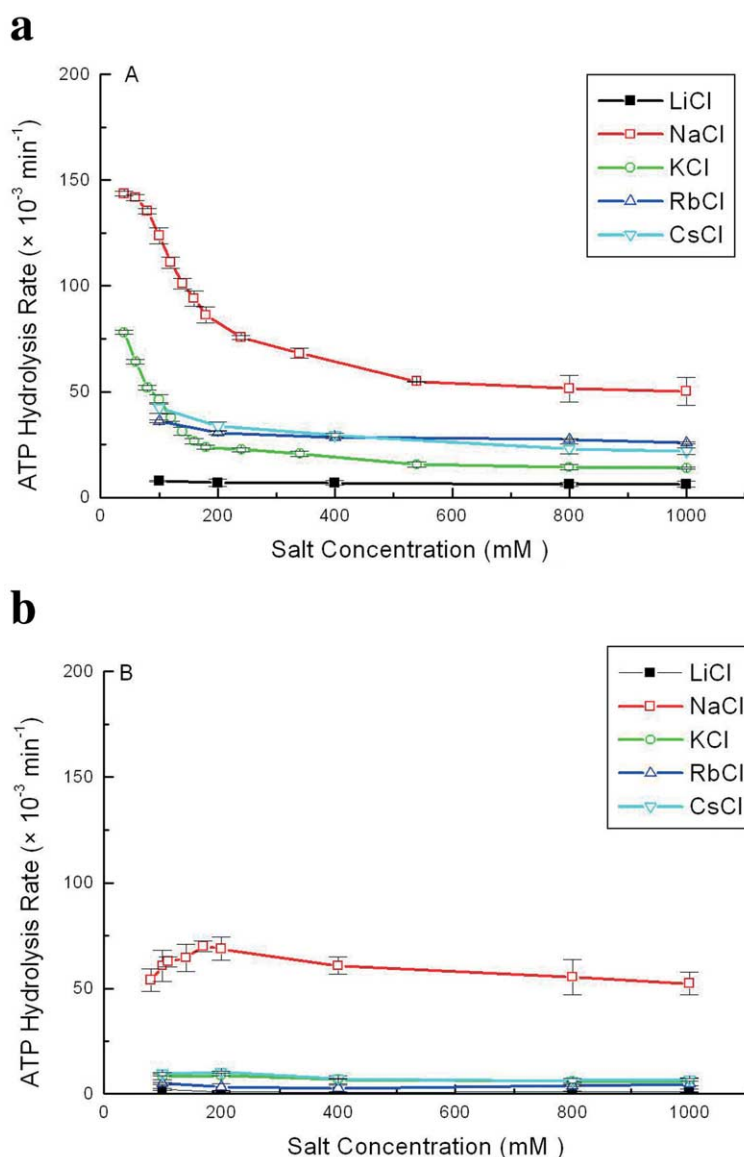


Fig. 1. The ATPase activity of the WT and A100P mutant MutL. a and b: ATP hydrolysis rate of WT (a) and the A100P mutant (b) MutL in the presence of varying amounts of LiCl, NaCl, KCl, RbCl, and CsCl. The lowest NaCl and KCl concentrations used in the ATPase activity assay were 50 mM, below which MutL aggregates.

that this cation-binding site must be occupied by either  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Rb}^+$  or  $\text{Cs}^+$  for MutL to bind and hydrolyze ATP as in the case of BCK.  $\text{Li}^+$ , which is probably too small to fill the site, does not support the MutL ATPase activity.

### 3.2. Structural and enzymatic analysis of the A100P mutant MutL

Structural comparison of MutL and BCK reveals an obvious difference in the monovalent cation-binding site. Pro341 of BCK, which makes close van der Waals contacts (3.4–3.6 Å) with the bound  $\text{K}^+$ , is replaced by Ala100 in MutL. The small alanine side chain leaves a cavity adjacent to the  $\text{K}^+$ -binding site in MutL, which allows the entry of a water molecule (Fig. 2a). To examine the role of residues surrounding the  $\text{K}^+$ -binding site, we mutated Ala100 of MutL to Pro. The A100P mutant MutL has two-fold reduced ATPase activity as compared with WT MutL at the physiological salt concentrations,  $\sim 100$  mM NaCl (Fig. 1b). Interestingly, the salt con-

centration for the maximal ATPase activity is  $\sim 50$  mM for WT MutL and increased to be  $\sim 150$  mM for the A100P mutant, but both proteins exhibit comparable ATP hydrolysis rates at NaCl concentrations above 200 mM (Fig. 1). This indicates that the reduced ATPase activity of the A100P mutant MutL at low salt concentrations is due to its reduced affinity for  $\text{Na}^+$ . When the NaCl concentration is high enough to keep the monovalent cation site occupied, the mutant and WT MutL show no difference in their ATPase activity. Parallel to the reduced affinity for  $\text{Na}^+$ , the A100P mutant MutL also appears to absolutely depend on sodium for the ATPase activity (Fig. 1b).

Crystals of the LN40(A100P)-ADPnP complex were obtained in the presence of 400 mM KCl and are isomorphous to the WT crystals. Anomalous diffraction data were collected, but no anomalous peak was found in the expected potassium-binding site. A positive peak in a  $2F_o - F_c$  electron density map indicated an atom occupying the same site as the

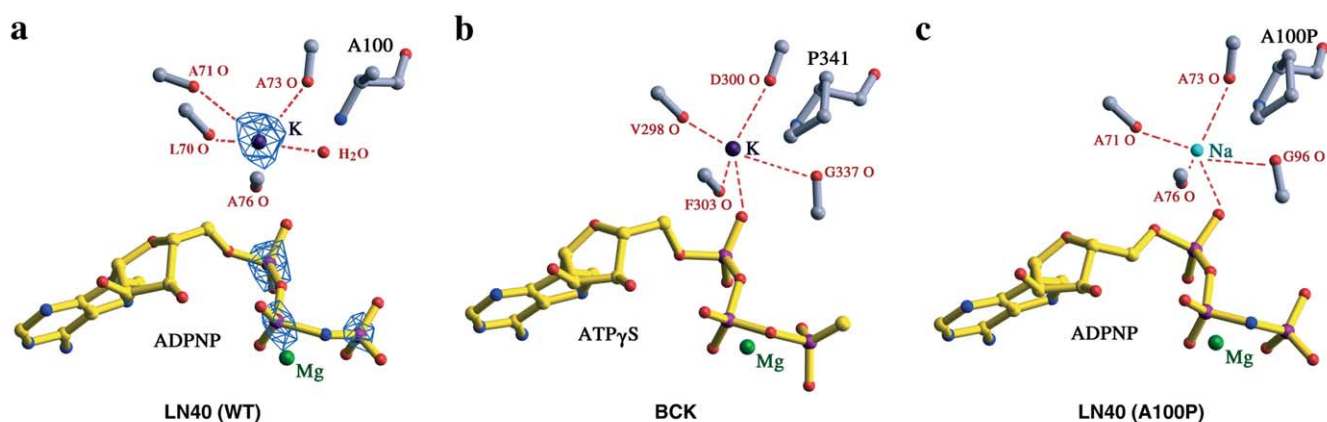


Fig. 2. Crystal structures of the ATP-binding site of the WT LN40, BCK and LN40(A100P). a: The active site configuration of the LN40–ADPNP complex in the presence of  $\text{Mg}^{2+}$  and  $\text{K}^+$ . Peaks above  $3\sigma$  in an anomalous difference Fourier map are contoured in light blue. b: The  $\text{ATP}\gamma\text{S}$  bound BCK. c: The active site configuration of the LN40(A100P)–ADPNP complex. The ATP analogs (ADPNP and  $\text{ATP}\gamma\text{S}$ ) are shown in yellow bonds, the protein carbonyl groups in gray bonds, the  $\text{Mg}^{2+}$  ion is shown in dark green,  $\text{Na}^+$  ion in light blue, and  $\text{K}^+$  ion in dark purple. Coordination of the monovalent cation is highlighted in dark pink.

$\text{K}^+$  in WT LN40. Its coordination by four carbonyl oxygen atoms is superimposable to that of the  $\text{K}^+$  ion in BCK (Figs. 2 and 3). Three of the carbonyl oxygen atoms (residues 71, 73 and 76) are located at the N-terminal hinge of the ATP lid, the fourth carbonyl oxygen (residue 96) is from an adjacent loop at the C-terminal hinge of the ATP lid. The water molecule that serves as the fifth ligand to the  $\text{K}^+$  in the WT

LN40–ADPNP structure is eliminated in the LN40(A100P)–ADPNP complex because of the proline substitution, and the non-bridging oxygen from the ADPNP  $\alpha$ -phosphate serves as the fifth ligand as in BCK (Fig. 2). We conclude that the monovalent cation site in the LN40(A100P)–ADPNP complex is occupied by a  $\text{Na}^+$  ion based on the geometry of coordination, inter-atomic distances (2.8–3.0 Å), valence [24], the

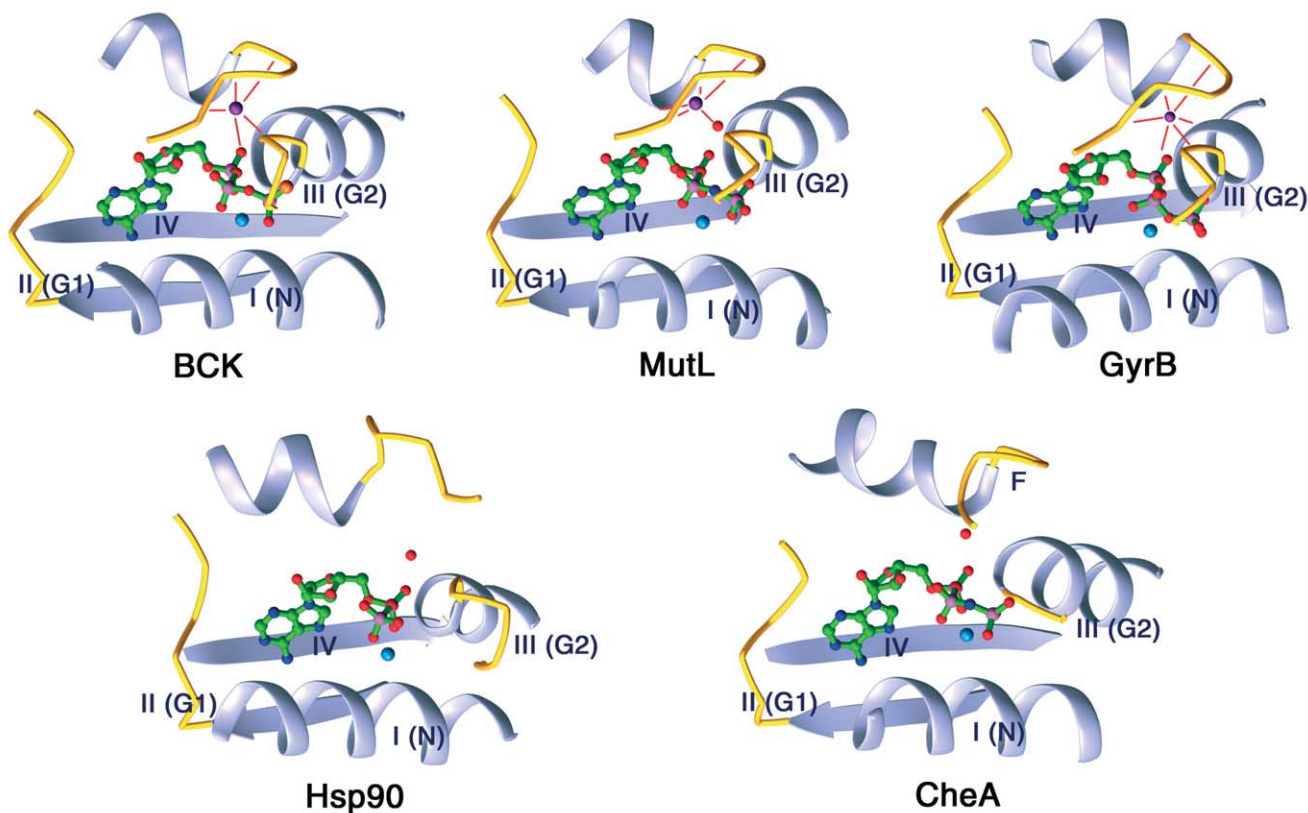


Fig. 3. Comparison of the monovalent ion-binding site of BCK, MutL, GyrB, human Hsp90 and CheA. Secondary structures surrounding the ATP-binding site are shown in gray ribbon drawings including motifs I (N) and IV; motif II (G1) and the N- and C-terminal (motif III) hinges of ATP lid are drawn in yellow. For clarity, the connecting part of the ATP lid is not shown. The bound nucleotides are shown in ball-and-stick models, divalent and monovalent cations are shown as cyan and purple spheres, and water oxygens as red sphere.



lack of anomalous signal, electron density, and the ATPase activity analysis. The Na<sup>+</sup> ion was probably carried over from the NaCl used during protein purification and complex formation with ADPnP and Mg<sup>2+</sup> but resisted replacement by K<sup>+</sup> afterwards during buffer exchanges and crystal soaking. The preferred and tight binding of Na<sup>+</sup> ion compared with WT protein is consistent with the Na<sup>+</sup>-dependent ATPase activity of MutL(A100P).

### 3.3. Monovalent cation binding by other GHKL proteins

Our comparison of the structural and catalytic properties of BCK and MutL revealed a common monovalent cation-binding site, which is exclusively occupied by K<sup>+</sup> in BCK, and by either Na<sup>+</sup> or K<sup>+</sup> in MutL. In the hinges of the ATP lid, a structurally conserved tight turn with the preceding  $\alpha$ -helix and an invariable Gly in motif III provide four carbonyl oxygen atoms that coordinate the monovalent cation, which in turn coordinates the ATP lid formation and facilitates ATP binding in both BCK and MutL (Figs. 2 and 3). A crystal structure of the gyrase ATPase fragment (GyrB) refined to 2.0 Å (R.J. Lewis and D.B. Wigley, personal communication) reveals a monovalent cation-binding site coordinated by the same set of four carbonyl oxygen atoms, the  $\alpha$ -phosphate of the nucleotide, and a Ser side chain (Ser212), which is equivalent to Pro341 of BCK and Ala100 of MutL (Fig. 3). The cation is most likely Na<sup>+</sup> in the case of gyrase since potassium salts were not used during protein purification or crystallization.

Due to the absence of an ordered  $\gamma$ -phosphate of ATP, an ATP analog or the nucleophilic histidine residue, the ATP lid remains partially disordered in Hsp90 [7,25], and EnvZ and CheA histidine kinase [8,18]. In these cases, the hinges of the ATP lid that potentially coordinate the monovalent cation adopt different conformations from those observed in BCK, MutL and GyrB (Fig. 3), and no monovalent cation was found. The  $\alpha$ -phosphate of the bound nucleotide, however, is often associated with a water molecule (Fig. 3), which may be replaced by a monovalent cation when the ATP-binding site is fully formed.

The structural determinants of monovalent cation specificity are not immediately obvious from the 2.0–2.3 Å crystal structures of BCK, MutL and GyrB. Protein sequence conservation, however, provides a clue. Pro341, which is the  $n+4$  residue from the Gly that provides the fourth coordinating carbonyl oxygen from the G2 box (motif III), is conserved in almost all members of the mitochondrial serine kinases, including pyruvate dehydrogenase kinase [9]. In contrast, the equivalent Ala100 is not conserved in the MutL family. It is often replaced by Gly or Ser, or even Pro as in *S. pneumoniae* (HexB) [16]. Interestingly, in spite of exhibiting a Na<sup>+</sup>-dependent and somewhat reduced ATPase activity in vitro, the A100P mutant MutL retains the mismatch repair function in *Escherichia coli* (J.H. Miller, personal communication). The equivalent residue of Pro341 (BCK) and Ala100 (MutL) in type II topoisomerases is also variable and can be Ser, Thr or Lys [1]. The selection of potassium by BCK and related protein kinases may have developed in mitochondria, where K<sup>+</sup> is most abundant.

### 3.4. Conclusion

Based on the conservation of the protein secondary structure surrounding the monovalent cation-binding site, a mono-

valent cation associated with ATP binding may be a common feature of all GHKL proteins. The diversity of protein sequence at the position equivalent to Pro341 of BCK, Ala100 of MutL and Ser121 of GyrB may influence the specificity of monovalent cation selection. This position is occupied by a conserved residue in mitochondrial protein kinases (proline) and the Hsp90 (tyrosine), but by variable residues in MutL, type II topoisomerases and the bacterial histidine kinase families. The selective binding of monovalent cations by an individual protein or the collective members of a sub-family may be useful for drug design. Antibiotic and antitumor drugs currently targeting gyrase, topoisomerase and Hsp90, including coumarin derivatives, geldanamycin and radicicol, all bind near the adenine- and sugar-binding pocket without extending to the phosphate and cation-binding sites [13–15]. The involvement of monovalent cations in the active site of GHKL proteins can be exploited for designing nucleotide analogs that contain a modified  $\alpha$ -phosphate group mimicking a specific monovalent cation [26,27], and such nucleotide analogs may be used to specifically inhibit a small subset of GHKL enzymes and minimize cross-reactivity.

In a more general sense, given the ubiquitous existence of sodium and potassium ions in living organisms, it is not surprising to find a monovalent cation-binding site in the ATP-binding pocket of BCK, MutL and GyrB proteins. It is, however, unique that these monovalent cations are directly involved in active site formation, substrate binding and catalysis. Prior to the studies of BCK, monovalent cations are not noted for participating in active site formation and have only been reported to stabilize protein as well as DNA and RNA tertiary structures [28–32]. As observed here, small monovalent cations, particularly Na<sup>+</sup>, can be easily misidentified as water molecules. With careful examination, more monovalent cation-binding sites than reported thus far are likely to be identified for either tertiary structural formation or enzymatic function.

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